Phylogenetic analysis of Malagasy Gastrorchis and Phaius (Orchidaceae) based on internal transcribed spacer (ITS) sequence

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Phylogenetic analysis of Malagasy *Gastrorchis* and *Phaius* (Orchidaceae) based on internal transcribed spacer (ITS) sequence

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Abstract

The molecular phylogenetics among five species of *Gastrorchis* and *Phaius pulchellus* all endemic to Madagascar and additional four species of non-Malagasy *Phaius* was studied on the basis of the sequence analysis of ITS region of rDNA. The species of *Gastrorchis* and those of *Phaius* studied constituted two respective clades, excepting Malagasy *P. pulchellus* was placed in the clade of *Gastrorchis*. This fact suggests that Malagasy *P. pulchellus* might be shared and originated from the common ancestor of *Gastrorchis*.

Key words : *Gastrorchis*, ITS, molecular phylogenetics, *Phaius*.

The terrestrial or epiphytic genera *Gastrorchis* Schlct. and *Phaius* Lour. (Orchidaceae) are taxonomically placed in the subfamily Epidendroideae, the tribe Arethuseae, sub-tribe Bletiinae (Dressler 1982). *Gastrorchis* endemic to Madagascar (Schlechter 1825) consists of six species according to Perrier de la Bathie (1939–1941) or nine species and three varieties according to Du Puy et al. (1999). *Phaius* consists of approximately 50 species and is distributed from Africa, east to Asia down to Polynesia (Perrier de la Bathie 1939–1941 ; Ohwi 1965). However, *Phaius pulchellus* Kraenzl is only the species in the genus found in Madagascar as well as French Reunion (Bosser 1971 ; Du Puy et al. 1999).

Gastrorchis has been firstly considered to be placed as the subgenus in *Phaius* in the Orchidaceae (Blume 1858) and then, has been treated as the distinct genus isolated from *Phaius* (Perrier de la Bathie 1939–1941). Moreover, *Gastrorchis* has been once treated as a section in *Phaius* (Summerhayes 1964). More recently, *Gastrorchis* and *Phaius* have been taxonomically revised to be separate genera in the subtribe Bletiinae by Dressler (1982). Malagasy *Gastrorchis* and *Phaius* were easily identified by some morphological characters ; e.g., the former genus had no spur and no fusion of the base of the lip with the column (Hermans 1999).

Only a karyomorphological approach in *Gastrorchis* and *Phaius* is available up to date in most standard references (Faliniaina and Kondo 2003) : Four species and one variety of *Gastrorchis* and *Phaius pulchellus* and its variety had common karyomorphological characteristics and the chromosome number of 2n=40 and thus, they seem to closely related to each other. These karyomorphological similarities support Dressler's hypothesis (1982) that *Gastrorchis* and *Phaius* seem to be closely related to each other on the basis of morphological characters.

The nucleotide sequences of the internal transcribed spacer 1 and 2 (ITS 1 and ITS 2) from the nuclear ribosomal DNA (nrDNA) region are widely used in molecular phylogenetics and systematics at the species and genus levels (Baldwin et al. 1995). The ITS regions have been well -used in phylogenetic analysis in orchidaceous plants (Whitten et al. 2000 ; Pridgeon et al. 2001 ; Gravendeel et al. 2001 ; Salazar et al. 2003 ; Tsai et al. 2004 ; Van Den Berg et al. 2005). However, any molecular phylogeny has not yet been applied to *Gastrorchis*, *Phaius* and their close relatives of sub-tribe Bletiinae.

In this paper, we attempt to determine genetic and systematic relationship between Malagasy *Gastrorchis* and *Phaius pulchellus* based on the sequence analysis of ITS region.

Materials and methods

Plant materials :

Four species and one variety of *Gastrorchis* and one species and one variety of *Phaius* in Madagascar were used in this study (Table 1). For comparison, *Calanthe sylvatica* (Thouars) Lindl. in Madagascar was chosen and used as the outgroup since it was taxonomically closely related to *Gastrorchis* and *Phaius* placed in the subtribe Bletiinae and shared same geographical localities and habitats (Dressler 1982). They were collected and cultivated in Laboratory of Plant Chromosome and Gene Stock, Graduate School of Science, Hiroshima University, Japan. Additional four species of *Phaius* in Indonesia, Japan, French New Caledonia and American Samoa, respectively were collected and cultivated in Hiroshima Botanical Garden.

DNA extraction, amplification, cloning and sequencing :

Total DNA of each of the studied taxa was extracted from fresh leaves using CTAB method (Doyle and Doyle 1987). The nucleotide sequences of ITS 1, 5.8 S rDNA and ITS 2 (ITS region) were amplified from total DNA using ITS 5 (5'-GGAAGTAAAAGTCG-TAACAAGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') PCR primers (White et al. 1990). The PCR amplifications were performed in a GeneAmp PCR system 2700 (Perkin-Elmer, Boston, MA, USA) thermal cycler for an initial 1 min denaturation at 97℃, then 27 cycles of 1 min at 97℃, 1 min at 52℃, and 2 min at 72℃ followed by an extention period of 7 min at 72℃. The ITS PCR products were purified by the High Pure PCR Product Purification Kit (Roche, Indianapolis, IN, USA) and cloned by the pGEM-T easy vector system (Promega, Madison, WI, USA) because the peaks of direct sequencing data of ITS PCR products were broad or overlapped and they were impossible to be analyzed. Ligation and transformation were prepared according to the manufacturer's protocol. Transformed *Escherichia coli* strain JM 109 (TaKaRa Bio, Otsu, Japan) was spread onto LB agar plate (LB medium, including 100 mg/ml ampicillin, 0.5 mM IPTG, 40 μg/ml X-Gal) for blue/white selection and incubated at 37℃ overnight. Between 5-10 white colonies of each species were picked up at random and checked presence of ITS region by PCR described above. At least four clones of each species with ITS region were incubated overnight in LB medium including 100 mg/ml ampicillin and plasmid DNA were extracted and purified. The ITS regions were sequenced by ABI 377 automated sequencer using Big Dye Terminator kit (ABI, Foster city, CA, USA) following the manufacturer's protocol. Sequencing primer was ITS 4.

Data analysis :

The sequences obtained were aligned with CLUSTAL W program (Thompson et al. 1994) on the DDBJ website (http : //www.ddbj.nig.ac.jp). The phylogenetic tree was constructed with a parsimony method using PAUP 4.0 b program (Swofford 2000). The heuristic search was conducted with 1,000 random addition replicates, rearrangements limited to 100,000 per replicates, tree bisection-reconnection (TBR) and MulTree on. The bootstrap analysis was carried out on 1,000 replicates using the heuristic search option in PAUP (Felsenstein 1985). To quantify the diversity of ITS sequences, the number of polymorphic nucleotide sites per nucleotide site was calculated (Nei 1987).

Results and discussion

Nucleotide sequence analysis and comparison

Amplification of ITS regions using the primer pair described above gave PCR products of approximately 750 bp in length. The sequences of them were comprised of a partial sequence of 18 S rRNA gene, the ITS region, and a partial sequence of 26S rRNA sequence. The boundaries of the ITS 1 and ITS2 and nuclear rDNA coding regions were determined by comparison with an almost complete sequence for the 18S–26S nrDNA cistron of *Arabidopsis thaliana* (Accession No. X 52320). The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank databases under accession numbers AB 222023 to 222034 and AB 239254 to

AB 239295. The length of ITS region in our samples varied from 229–244 bp for the ITS 1 region, 167 bp for the 5.8S rRNA gene, and 255–262 bp for the ITS 2 region (Table 1). Their intra-individual polymorphism was confirmed in all species in this study: One to 11 polymorphic sites and 1–4 indels between respective clones were found in each species and some of their clones had same sequence (Table 1). These clones of the same sequences were treated here as one common clone following the phylogenetic analysis.

ITS sequences of the five species of *Gastrorchis* (17 clones), six species of *Phaius* (23 clones) and two species of *Calanthe* (5 clones) as the outgroup were aligned and yielded 670 characters. Percentage of polymorphic sites of the ITS region excluding the indels within 17 taxa of *Gastrorchis* was 8.3%, that between 23 taxa of *Gastrorchis* and Malagasy *Phaius* was 9.6%, that between 40 taxa of *Gastrorchis*, Malagasy *Phaius* and non-Malagasy *Phaius* was 25.3%, that between 45 taxa of *Gastrorchis*, *Phaius* and *Calanthe* (outgroup) was 29.3%, respectively (Table 2).

Phylogenetic analysis

Maximum parsimony analysis of the ITS data produced 89186 equally parsimonious trees of 282 steps with a consistency index including uninformative characters (CI) of 0.816 and a retention index (RI) of 0.965. Same topology was yielded by MP analysis with indels or without indels. Thus, the strict consensus tree with indels was drawn in Fig. 1. The phylogram showed two major clades; one consisted of the Malagasy taxa of *Phaius* and *Gastrorchis* and the other consisted of non-Malagasy *Phaius*. The monophyly of Malagasy clade of *Gastrorchis* and *Phaius pulchellus* was supported by 100% bootstrap values (BS), while the monophyly of non-Malagasy *Phaius* was supported by 63.4% BS. This result suggested that the genus *Phaius* was polyphyletic taxon, and Malagasy *Phaius* was differentiated from a part of the clade of *Gastrorchis*.

In the Malagasy clade of *Gastrorchis* and *Phaius pulchellus*, a subclade was inferred with 68.8% BS, which included *G. humblotii* (Rchb. f) Schltr. var. *schlechteri* (H. Perrier) Senghas ex J. Bosser et P.J. Cribb, *G. lutea* (Ursch et Toill.

Table 1. The materials used in this study and intra-individual variation in ITS region of *Gastrorchis*, *Phaius* and *Calanthe* species

Abbreviation	Species	Voucher specimen and source country	No. of clones	Nucleotide substitution	Indel	ITS length (bp)	Database accession
G. fran	*Gastrorchis francoisii* Schltr.	LPCGS-705: Madagascar	3	3	0	662	AB222023, AB239254-55
G. humb	*Gastrorchis humblotii* (Rchb.f.) Schltr.	LPCGS-706: Madagascar	3	2	0	660	AB222024, AB239258-59
G. humb. sch	*Gastrorchis humblotii* var. *schlechteri* (H. Perrier) Senghas ex Bosser and P.J. Cribb	LPCGS-707: Madagascar	5	7	1	670-671	AB222025, AB239262-65
G. tube	*Gastrorchis tuberculosa* (Touars) Schltr.	LPCGS-708: Madagascar	2	5	0	669	AB222026, AB239267
G. lute	*Gastrorchis lutea* (Ursch and Toill.Gen. ex Bosser)	LPCGS-709: Madagascar	4	3	0	669	AB222027, AB239269-71
P. pul. pul	*Phaius pulchellus* var. *pulchellus* Kraenzl.	LPCGS-710: Madagascar	4	4	4	664-668	AB222028, AB239273-75
P. pul. sand	*Phaius pulchellus* var. *sandrangatensis* Bosser	LPCGS-711: Madagascar	2	4	0	665	AB222029, AB239276
P. ambo	*Phaius amboinensis* Blume	8599-1: Indonesia	5	11	1	654	AB222030, AB239279-82
P. mino	*Phaius minor* Blume	8479: Japan	3	3	0	653	AB222031, AB239283-84
P. tanc	*Phaius tancarvilleae* (Banks ex L'Her.) Blume	7587-1: French New Caledonia	5	6	3	656-659	AB222032, AB239286-89
P. grae	*Phaius graeffei* Rchb. f.	10158: American Samoa	3	2	0	656	AB222033, AB239290-91
C. sylv	*Calanthe sylvatica* (Touars) Lindl.	LPCGS-712: Madagascar	4	1	2	656-657	AB222034, AB239293-95
P. mino_4	*Phaius minor* Blume	Van Den Berg et al. 2005				657	AF521051
C. alis	*Calanthe alismaefolia* Lindl.	Liao et al. 2005				654	AY882615

Table 2. Percentage of polymorphisms * among *Gastrorchis*, Malagasy *Phaius* and non-Malagasy *Phaius*

Group	ITS 1 (%)	5.8 S (%)	ITS 2 (%)	Total (%)
within *Gastrorchis*	14.0	1.8	7.4	8.3
Gastrorchis and Malagasy *Phaius*	15.4	3.6	8.1	9.6
Gastrorchis, Malagasy *Phaius and* non-Malagasy *Phaius*	36.1	9.7	25.6	25.3
Gastrorchis, *Phaius* and *Calanthe* (outgroup)	40.9	9.7	32.0	29.3

* The percentages were calculated by Nei (1987)

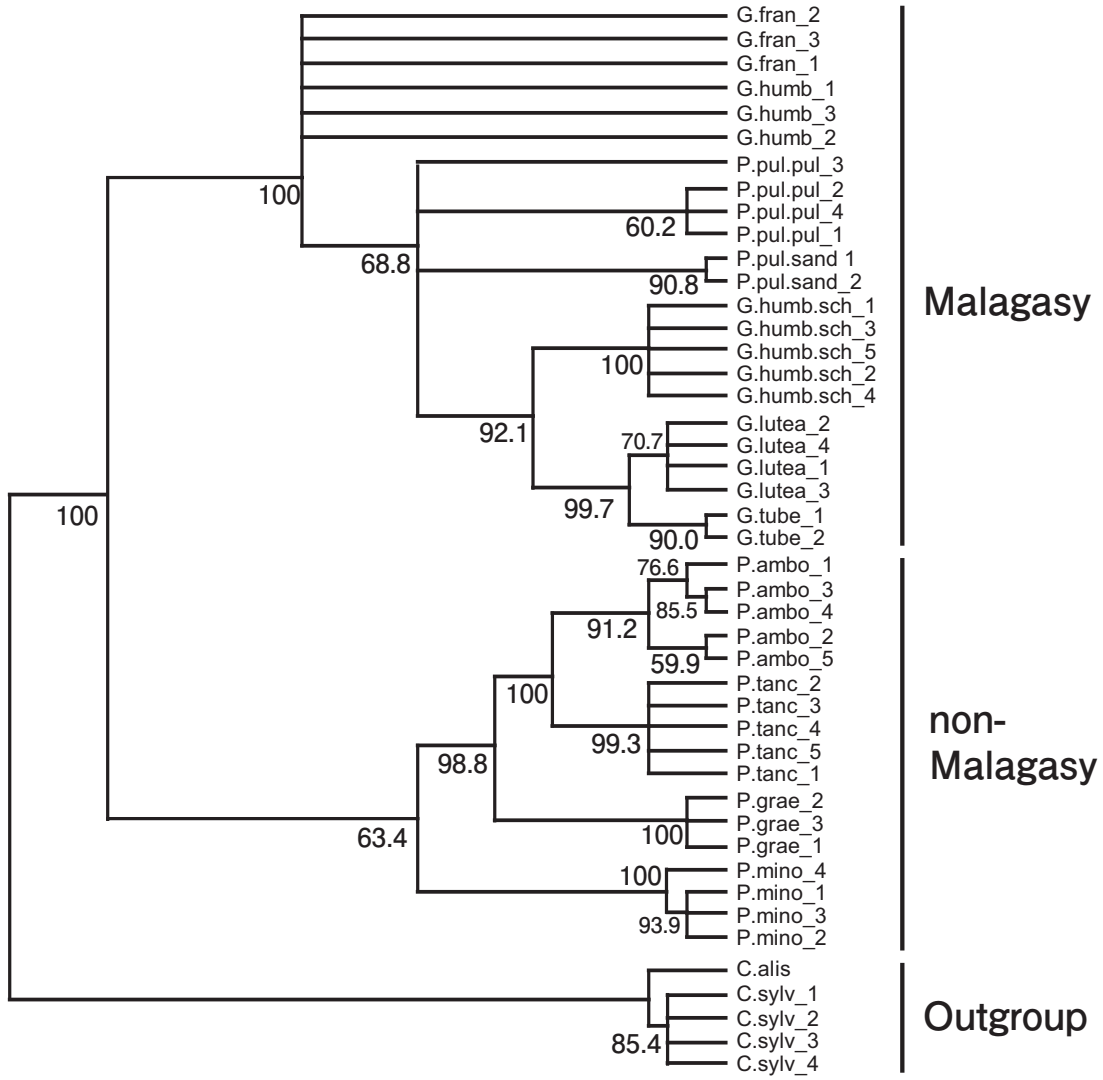

Fig. 1. The strict consensus tree of 89186 most parsimonious trees based on the internal transcribed spacer (ITS) sequence data (Tree length=282, CI=0.816. RI=0.965). Numbers listed along the branch show bootstrap values. See Table 1 for explanation of the abbreviations.

-Gen. ex Bosser) Senghas and *G*. *tuberculosa* (Thouars) Schltr, *P*. *pulchellus* var. *pulchellus* and *P*. *pulchellus* var. *sandrangatensis* J. Bosser, and the others included *G*. *francoisii* Schltr. and *G*. *humblotii*. In the subclade, *G*. *humblotii* var. *schlechteri*, *G*. *lutea* and *G*. *tuberculosa* made a clade supported by 92.1% BS. This fact suggested that the species of *G*. *humblotii* was not monophyletic, and both *G*. *humblotii* and *G*. *humblotii* var. *schlechteri* were originated from different ancestor, respectively. Thus, revision of the taxonomic position of the *G*. *humblotii* might be needed.

In spite of the Malagasy *Phaius pulchellus* having common flower characters with the non-Malagasy *Phaius*, the present results suggested that the Malagasy *P*. *pulchellus* was placed in the *Gastrorchis* cluster (100% BS; Fig. 1). The possibility for the incongruence between morphology and ITS sequence data could be convergent evolution or gene transfer through introgressive hybridization because *Gastrorchis* and *Phaius* could easily make hybrid (Dressler 1982). Although the data in this paper were not enough

to solve this problem, yet they indicated at least *Phaius pulchellus* was not voyaged from somewhere else but evolved in Madagascar.

Many more molecular analyses using not only genomic DNA but also plastid DNA as much as chloroplast DNA and/or mitochondria DNA sequences should be made for clarification, justification and revision of the relationship between *Gastrorchis*, Malagasy *Phaius* and non-Malagasy *Phaius*.

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増田　優・ファリニアイナ　ルシエン・近藤勝彦：マダガスカル産ラン科 *Gastrorchis* と *Phaius* の分子系統学的関係

マダガスカル固有の *Gastrorchis* 5 種と *Phaius pulchellus* とその 1 変種，マダガスカル以外に分布する *Phaius* 4 種，そして outgroup として両属に近縁な *Calanthe sylvatica* を用いて，系統学的関係の分析を行った。本研究では，それぞれの核リボソーマル DNA の ITS 領域を PAUP ver. 4.0 プログラムを用いて解析した。その結果，作成した分子系統樹から，マダガスカル固有の *Gastrorchis* と *Phaius pulchellus* は同じクレードに属し，マダガスカル以外に分布する *Phaius* は，異なるクレードに属することが明らかとなった。このことは，マダガスカルに固有の *Phaius pulchellus* が *Gastrorchis* との共通の祖先から派生したこと，マダガスカル以外に分布する *Phaius* とは別系統であることを示唆している。

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